

# Gender affects $^{13}\text{C}$ -ketoisocaproic acid breath test

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**Abstract. – Background and Aims:**  $^{13}\text{C}$ -Ketoisocaproic Acid Breath Test ( $^{13}\text{C}$ -Kica-BT) has been proposed to assess mitochondrial function. Aim of this study is to evaluate whether gender affects mitochondrial oxidation by means of  $^{13}\text{C}$ -Kica-BT in healthy subjects in basal conditions and after an acute oxidative stress induced by ethanol.

**Methodology:** 50 healthy volunteers were given 1 mg/kg of  $^{13}\text{C}$ -Kica together with 20 mg/kg of L-leucine dissolved in 200 ml of orange juice. Breath samples were taken at baseline, every 5 minutes for 45 minutes and then every 15 minutes until 2 hours. Forty-eight hours later the test was repeated 30 min after ethanol ingestion (0.5 g/kg body weight).  $^{13}\text{CO}_2$  enrichment in breath was analyzed by isotope ratio/mass spectrometry. Statistical analysis was performed using the student's t test.

**Results:** At baseline conditions, the percentage of Ketoisocaproic acid in 2 hours was significantly higher in females than in males. Ethanol significantly reduces the oxidation of Ketoisocaproic acid. Conversely, no differences were observed between groups after the ethanol oral load.

**Conclusions:** Decarboxylation of  $^{13}\text{C}$ -Kica was significantly higher in females than in males. Ethanol decreases Kica decarboxylation in particular in women. Further studies remain needed to establish whether sexual hormones could interfere with the metabolism of Kica.

*Key Words:*

Gender, Ethanol, Kica breath test.

## Abbreviations

$^{13}\text{C}$ -Kica-BT =  $^{13}\text{C}$ -Ketoisocaproic Acid Breath Test

BCKA = Branched-chain  $\alpha$ -ketoacids

Kica = Ketoisocaproic acid

BMI = Body mass index

$\%^{13}\text{C}$  = Percentage of tracer oxidated over the test period

$\%^{13}\text{C}$ -dose/h = Percentage of the dose of  $^{13}\text{C}$  administered recovered per hour

$^{13}\text{C}$ -peak = Time of the peak exhalation

## Introduction

Liver mitochondria are essential for hepatocyte function, representing the major source of energy through ATP synthesis. In particular, the function of electron transport chain was found to be impaired both in acute and chronic liver diseases<sup>1</sup>, leading to decreased oxidative metabolism of various substrates and to impaired recovery of the hepatic energy state after a metabolic insult. Interestingly, the development of hepatocellular steatosis in chronic alcohol abusers was consequence of reduction of mitochondrial lipoperoxidations<sup>2</sup>. Therefore, mitochondrial function assessment might represent an important tool for the management of patients with liver disease. However, tests available to explore mitochondrial activity are complex and often invasive<sup>3</sup>. Breath testing is a safe and non-invasive tool for investigating the integrity of different metabolic pathways by means of  $^{13}\text{C}$ -labeled substrates<sup>4</sup>. Tests based on substrates producing  $\text{CO}_2$  during hepatic mitochondrial metabolism, such as branched-chain  $\alpha$ -ketoacids (BCKA), could give a dynamic analysis of liver mitochondrial function and have been proposed in the last decade to assess mitochondrial function. In particular, the decarboxylation of ketoisocaproic acid (Kica), occurring almost completely in hepatic mitochondria is the first step of metabolism that finally produces  $\text{CO}_2$ <sup>3</sup>.  $^{13}\text{C}$ -Kica breath test was first proposed to assess mitochondrial function in rats<sup>5</sup>. Then it was proposed as a specific marker of ethanol intake in chronic ethanol

abusers and to differentiate between alcoholic and non alcoholic steatosis in men<sup>6-9</sup>. Finally, the <sup>13</sup>C-Kica breath test was used to assess the effect of a number of xenobiotics on the activity of mitochondrial dehydrogenase<sup>10-12</sup>. The effect of ethanol on liver and gastric dehydrogenase was not well established yet because regulation of such activity was complex and difficult to evaluate<sup>8</sup>. In particular, studies carried out on mice and humans have found difference in gastric and liver dehydrogenase activity between males and females<sup>13-15</sup>. At present, however, it has not been clearly described whether <sup>13</sup>C-Kica breath test is affected by gender even if an increased metabolism of Kica has been described in a recent study, performed on a low number of subjects<sup>16</sup>. Aim of our study is to explore the relations between gender, ethanol and liver mitochondrial function in young healthy subjects by means of <sup>13</sup>C-Kica-BT.

## Methods

Fifty healthy volunteers (mean age  $26 \pm 2$ , BMI 22.82), 25 males (mean age  $25.7 \pm 3$ , BMI 23.12) and 25 females (mean age  $26.8 \pm 2$ , BMI 22.54), were enrolled in the study. Informed consent was obtained by each subject and the study was approved by our local Ethics Committee. The inclusion criteria were:

1. Assumption of ethanol < 30 g/week
2. BMI within the 10% of ideal body weight
3. No history of liver, cardiovascular and metabolic diseases
4. No assumption of any kind of medications in the last 2 months.

All subject performed an abdominal liver ultrasonography and classical blood test for liver function (serum aminotransferase, bilirubin, PT, albumin, alkaline phosphatase and gamma-glutamyltranspeptidase) to exclude the presence of liver and gall bladder diseases.

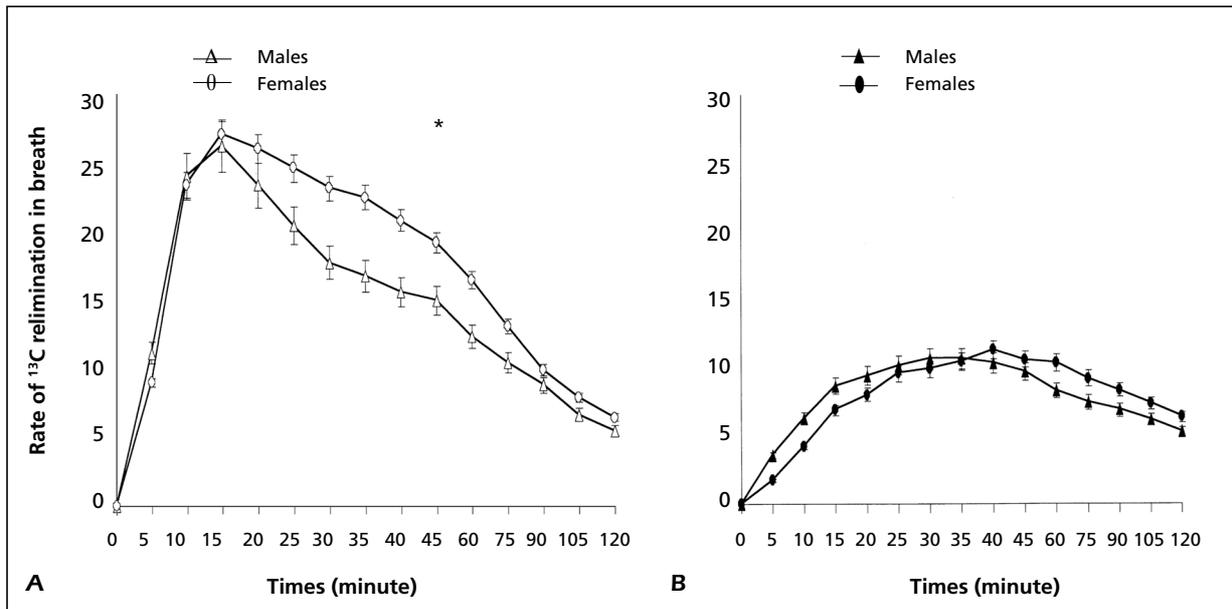
After an overnight fasting all subjects performed <sup>13</sup>C-Kica breath test in according to previous studies<sup>9</sup>. Baseline breath samples were collected by a straw in two test tube. Subjects then received 1 mg/kg of body weight of <sup>13</sup>C-Kica (4-methyl-2-oxopentanoic acid-1-<sup>13</sup>C sodium salt, 99% atom isoenrichment, Isotec, Miamisburg, Ohio) and 20 mg/kg of body weight of L-leucine dissolved in 200 ml of orange juice. The

leucine was administered to increase Kica decarboxylation reducing the transamination of Kica into leucine. Breath samples were collected every 5 minutes for 45 minutes and then every 15 minutes until two hours. Subjects remained at rest in seated position during the test. Forty-eight-hour later, the same protocol was repeated after an oral intake of 0.5 g/kg of ethanol. Breath samples were analyzed by an isotope ratio mass spectrometer (Finnegan-MAT, Bremen, Germany) to measure the ratio of <sup>13</sup>CO<sub>2</sub>/<sup>12</sup>CO<sub>2</sub>. Results were expressed as the percentage of tracer oxidated over the test period (%<sup>13</sup>C), as the percentage of the dose of <sup>13</sup>C administered recovered per hour (%<sup>13</sup>C-dose/h) and as the time of the peak exhalation (<sup>13</sup>C-peak). Results were expressed as mean  $\pm$  standard deviation. In figures results were expressed as mean  $\pm$  standard error. Statistical analysis was performed using the Student's t test for paired or unpaired data where appropriate.

## Results

Basal conditions: a peak in the exhalation of <sup>13</sup>CO<sub>2</sub> was found between 10 and 30 minutes after the <sup>13</sup>C-Kica administration with a slight but significant difference between groups (females  $19.6 \pm 8$  minutes; males  $14.2 \pm 4$  minutes;  $p < 0.05$ ) (Figure 1A). Conversely, the oxidation rate at peak time was similar between groups ( $22.7 \pm 5.7$  vs  $21.9 \pm 6.4$ ;  $p = ns$ ). Significant differences between sex considering the % of tracer metabolized were observed from 40 to 120 minutes and it was significantly higher in females than in males (%<sup>13</sup>C at 40 minutes:  $16.6 \pm 2\%$  vs  $13.9 \pm 1.8\%$ ,  $p < 0.05$ ; %<sup>13</sup>C at 120 minutes:  $25.4 \pm 2.3\%$  vs  $21.06 \pm 3.4\%$ ,  $p < 0.0001$ ) (Figure 2A).

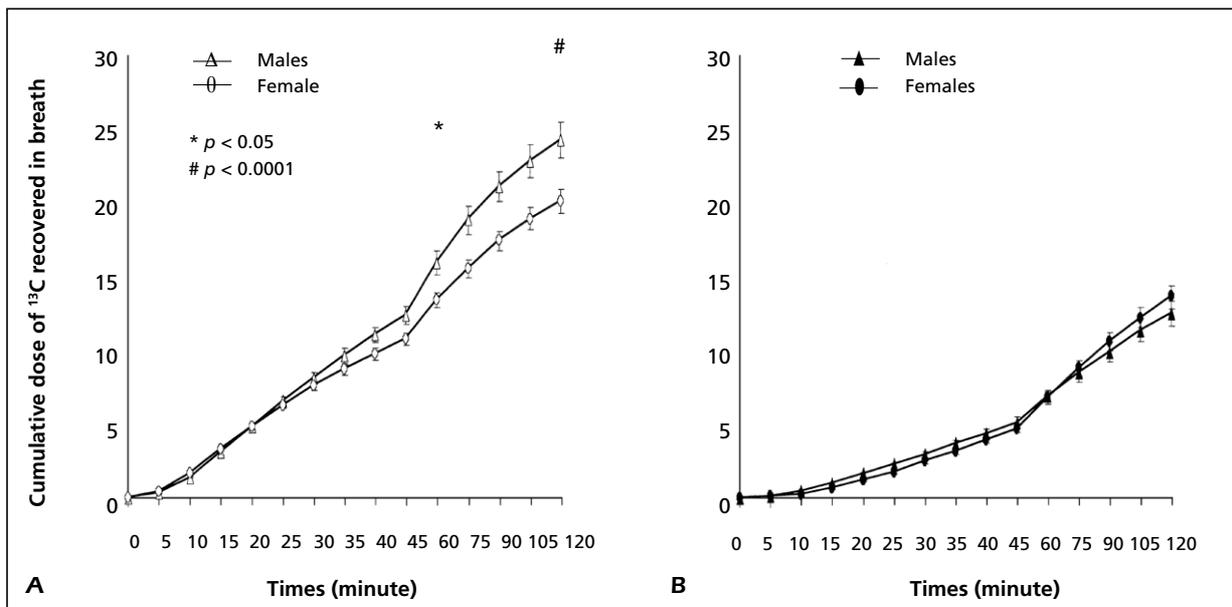
After ethanol intake a significant delay in peak appearance (female:  $19.6 \pm 8$  minutes vs  $43.2 \pm 12$  minutes;  $p < 0.001$ ; Figure 3A); males:  $14.2 \pm 4$  minutes vs  $46.4 \pm 33$  minutes;  $p < 0.0001$ , Figure 3B) and in oxidation rate at peak value (female:  $22.7 \pm 5.7$  vs  $9.3 \pm 2.6$   $p < 0.0001$ ; male:  $21.9 \pm 6.4$  vs  $8.8 \pm 4.7$ ;  $p < 0.001$ ; Figure 3A, 3B) were found in both groups. After ethanol oral load, no differences between groups were observed with regard to peak appearance (females:  $43.2 \pm 12$  minutes; males  $46.4 \pm 33$  minutes;  $p = ns$ ; Figure 1B) and oxidation rate at peak value ( $9.3 \pm 2.64$  vs  $8.8 \pm 4.7$ ;  $p = ns$ ; Figure 1B).



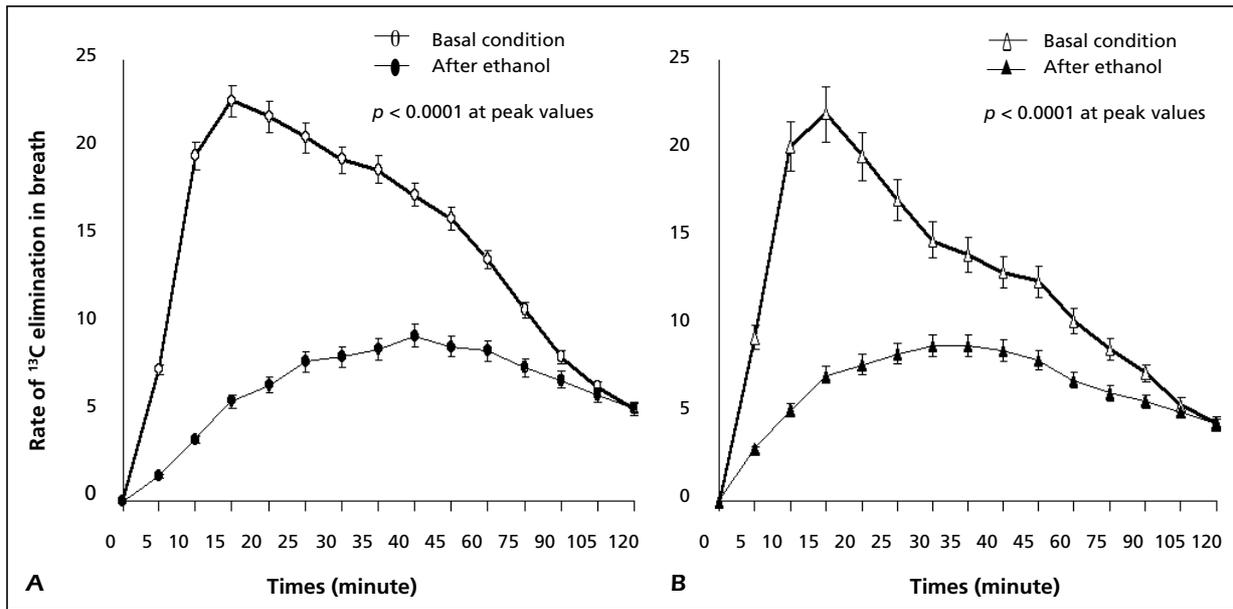
**Figure 1.** Kinetic curves of the time course of the <sup>13</sup>CO<sub>2</sub> exhalation during the Kica-BT in males and females in basal condition (**A**) and after ethanol load (**B**). (% <sup>13</sup>C-dose/h: percentage of the dose of <sup>13</sup>C administered recovered in breath per hour).

After ethanol ingestion the amount of %<sup>13</sup>C decreased significantly in both groups (Figure 4A, 4B). The reduction of oxydation of Kica was

higher in females (Figure 5), so that no differences in %<sup>13</sup>C were found between gender after ethanol assumption (Figure 2B).



**Figure 2.** Cumulative dose of <sup>13</sup>C recovered in breath during a Kica-BT in females and males at basal condition (**A**) and after ethanol load (**B**). (% <sup>13</sup>C: percentage of ketoisocaproic acid oxidation).

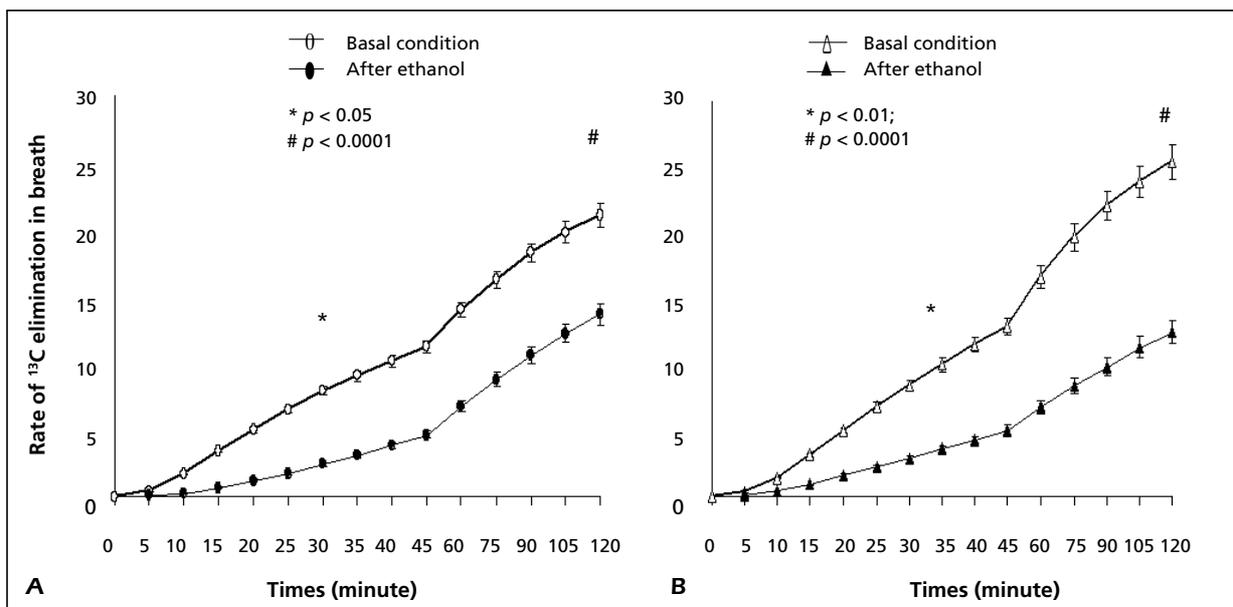


**Figure 3.** Kinetic curves of the time course of the  $^{13}\text{CO}_2$  exhalation during the Kica-BT in females (**A**) and males (**B**) in basal condition and after ethanol load. (%  $^{13}\text{C}$ : percentage of ketoisocaproic acid oxidation).

### Discussion

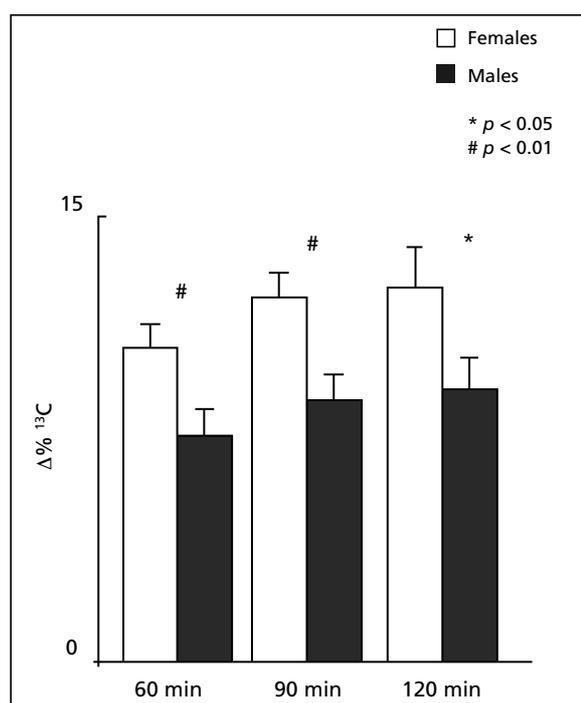
The  $^{13}\text{C}$ -Kica-BT has been demonstrated to be an effective, non invasive tool for the evaluation of liver mitochondrial function in humans. It has been used to study mitochondrial function in alco-

holic patients, in subject affected by macrovesicular steatosis, in case of reperfusion injury of the liver and after drugs or xenobiotics assumption<sup>3-5</sup>. Lauterburg et al firstly described the usefulness of Kica-BT to evaluate liver mitochondrial function in chronic ethanol abusers<sup>6</sup>. Bentsen et al



**Figure 4.** Cumulative dose of  $^{13}\text{C}$  recovered in breath during a Kica-BT in in females (**A**) and males (**B**) in basal condition and after ethanol load. (%  $^{13}\text{C}$ -dose/h: percentage of the dose of  $^{13}\text{C}$  administered recovered in breath per hour and: %  $^{13}\text{C}$ : percentage of ketoisocaproic acid oxidation).

**Figure 5.**  $\Delta\%$  cumulative dose at 60, 90 and 120 minutes before and after ethanol intake in males and females. ( $\Delta\%$ : % <sup>13</sup>C difference before and after ethanol intake of ketoisocaproic acid oxidation. Min: minutes).



and Parra et al observed that mitochondrial oxidation of Kica seems to be higher in female than in male healthy subjects<sup>9,16</sup> but the analysis was performed on a low number of subjects. Our study was performed on a higher number of healthy subjects and confirms previous data. Kica oxidation resulted lower and slower in males than in females. This difference was found only in basal condition, but not after an acute oral intake of ethanol. These results may be explained considering that the activity of the branched chain dehydrogenase may be regulated by hormonal factor through a reversible phosphorylation that could influence mitochondrial concentration of NAD<sup>+</sup>. The influence of sexual hormones on Kica enzymatic pathway should be further investigated.

Moreover, it seems that Kica metabolism is much more influenced by ethanol in female than in male subjects. We can speculate that the well known different activity of gastric and liver alcohol dehydrogenase between sex (lower in female) led to higher impairment of mitochondrial metabolism of Kica in women by means of higher ethanol blood levels<sup>13-15</sup>. Our data suggest that Kica-BT was an useful tool in assessing mitochondrial liver after an acute stress induced by ethanol but that sexual differences should be taken into account in future studies.

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